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Decompensation in hemorrhagic shock is the critical stage after which resuscitative efforts may prove futile. It is thus of interest to the military to be able to identify those casualties at risk for decompensation on the battlefield and to be able to assess response to resuscitative efforts. We hypothesize that decompensation results from potassium-mediated vasodilation and/or loss of cardiac contractility, and thus a method of measuring interstitial potassium should be a crucial part of future metabolic monitoring efforts. We propose a three-year plan of experimentation to assess variations of interstitial concentrations of potassium, lactate, pyruvate, glucose, calcium, and magnesium with the progression of hemorrhagic shock. The animal model to be used is controlled hemorrhage in rats. The method of microdialysis will be used to provide continuous monitoring of tissue composition in skeletal muscle and liver. These parameters will be compared to their corresponding serum concentrations and to hemodynamic parameters, cardiac contractility, and tissue levels of Na^+ - K^+ -ATPase. After establishing the methods in the model of hemorrhage, the effects of fluid resuscitation both in early and late stages of shock will be examined.

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INTRODUCTION

Decompensation in hemorrhagic shock is the critical stage after which resuscitative efforts may prove futile. It is of interest to the military to be able to identify those casualties at risk for decompensation on the battlefield and to be able to assess response to resuscitative efforts. We hypothesize that decompensation results from potassium-mediated vasodilation and/or loss of cardiac contractility, and thus a method of measuring interstitial potassium could be a crucial part of future metabolic monitoring efforts. We propose a three-year plan of experimentation to assess variations of interstitial concentrations of potassium, calcium, magnesium, lactate, pyruvate and glucose with the progression of hemorrhagic shock. The animal model to be used is controlled hemorrhage in rats. The method of microdialysis will be used to provide continuous monitoring of tissue composition in skeletal muscle and liver. These parameters will be compared to their corresponding serum concentrations and to hemodynamic parameters, cardiac contractility, and tissue levels of $\text{Na}^+\text{-K}^+\text{-ATPase}$. After establishing the methods in the model of hemorrhage, the effects of fluid resuscitation both in early and late stages of shock will be examined.

BODY

The following tasks composed the Statement of Work for Year One:

1. Purchase of major equipment, to include ICP-MS, microdialysis analyzer, blood gas analyzer, and refrigerated centrifuge. COMPLETED.

Purchased items included microdialysis analyzer (Model CMA 600, CMA/Microdialysis, North Chelmsford, MA), blood gas analyzer (i-STAT Portable Clinical Analyzer, i-STAT Corp., East Windsor, NJ), and refrigerated centrifuge (Sorvall RC5B+, Kendro Laboratory Products, Newtown, CT).

An ICP-MS analyzer was not purchased in accordance with the mandated reduction in the original budget. We instead have been able to obtain time on an analyzer at the Armed Forces Institute of Pathology (courtesy of LT John Ejnick, AFIP).

2. Expand current microdialysis protocol to include measurement of liver concentrations. COMPLETED.

Our surgical methods now include an additional one-inch midline abdominal incision. A microdialysis probe is placed into the central lobe of the liver through this incision, which is then closed. We have been able to do this without significant trauma to the liver or bleeding from the placement site. We do not observe that this abdominal incision and probe adversely affects the cardiopulmonary status of the animal during the experiment, as measured by comparison of arterial blood gases with those of previous animals which have not been instrumented in this way. At the end of the experiment, the placement site is carefully inspected to ensure that the probe maintained a secure position within the hepatic lobe, and this has been routinely true.

The three microdialysis probes (vein, muscle, and liver) are synchronously perfused using a three-syringe microinjection pump (Model CMA/102, CMA/Microdialysis) and the perfusate collected into a triple-probe sampling collector (Model CMA/140).

3. Establishment of ICP-MS analysis method for analysis of K^+ , Rb^+ , and natural isotopes of Ca^{2+} and Mg^{2+} from microdialysis samples. COMPLETED

The best method to correct for deviation from equilibrium in the microdialysis probes is the use of internal calibration standards. Suitable nonradioactive surrogates exist for the electrolytes of interest. The microdialysis perfusate was composed of $RbCl$ (4.5 mM), $^{44}CaCO_3$ (2.5 mM), and ^{26}MgO (6 mM). The isotopes of calcium and magnesium were purchased from Trace Sciences International (Ontario, CA).

Use of the DRC analyzer resulted in significant improvement in sensitivity to potassium and magnesium measurements compared to previous use of standard ICP instruments. There still remain problems with interference between calcium and the argon carrier, which restricts the ability to determine isotopic ratios for calcium. It is yet to be seen whether these problems can be overcome by technique changes with the available equipment.

4. Conduct experiments in 90 animals. 62 ANIMALS DONE.

The number of animals completed was lower than expected in part because our laboratory had to be shut down for a period of four weeks due to mandatory heating, ventilation and air conditioning maintenance work. We have refined and expanded our protocols to achieve nearly all desired technical objectives. Details on the methods may be found in the research proposal (MRMC Log No. 01155005). The experiments on the early decompensatory stage group (experiments stopped and tissues harvested at 25% return of the peak shed blood volume [PSBVR]) are nearly complete. Results to date are summarized as follows:

In vivo microdialysis probe equilibrium

For each internal calibration standard (Rb , ^{44}Ca , ^{26}Mg), the fractional equilibrium is given by $(c_p - c_d)/c_p$, where c_p is the concentration in the ingoing perfusate and c_d is the concentration in the outgoing dialysate. In Fig. 1, Rb fractional equilibrium (corresponding to K^+ equilibrium) for the blood vs. muscle and liver probes are shown. For potassium, the blood probes were relatively close to equilibrium (89% average over all times) in the intravascular space, while that for muscle and liver probes were reduced (61% and 70%, respectively). This suggests a significant unstirred boundary layers around probe in tissue, and emphasizes the need for the use of an internal standard for accurate absolute concentrations from microdialysis measurements. Calcium and magnesium equilibriums were reduced across the board compared to those for potassium (77, 66, and 68% for Ca ; 69, 49, and 55% for Mg). Lower microdialysis probe equilibriums for the divalent ions have also been observed by our lab *in vitro*. The explanation for this is unknown at present, but it suggests the possibility of electrostatic interactions interfering with ion transport.

Corrections for glucose, lactate, pyruvate, and glycerol normally require the use of radioactive isotopes and are difficult to perform *in vivo*. Reported values of fractional equilibrium for these larger molecules range from 15-40% (CMA/Microdialysis product information, CMA/Microdialysis, North Chelmsford, MA).

Potassium changes in muscle and liver vs. blood

In hemorrhaged animals, serum potassium showed a slow rise with time, while muscle potassium increases more rapidly and to a greater extent (Fig. 2). This early rise in muscle potassium during hemorrhage was not reflected in serum levels. In control animals, serum and muscle potassium were similar and constant with time. These relative trends are consistent with interstitial potassium elevations seen previously using other methods. (Illner and Shires, 1990; McKinley *et al.*, 1981).

The ratios of potassium concentration at 43 minutes of hemorrhage (approximate average time to peak shed blood volume) to those at baseline are shown in Table 1. Potassium levels in muscle of hemorrhaged animals were 1.98 times that of baseline ($p < 0.05$ compared to controls), while levels in blood and liver were not significantly changed. This specific elevation in muscle potassium may contribute to vasodilation in small resistance vessels leading to vascular decompensation.

Ca/Mg changes in muscle and liver vs. blood

As shown in Table 1, calcium and magnesium levels increased from baseline in most cases in vein and muscle, however, these changes were not significantly different from control animals.

Glucose, lactate, pyruvate, and glycerol changes in muscle and liver vs. blood

Glucose levels in blood, muscle, and liver all rose at 43 minutes of hemorrhage, but the change was significant from controls only in blood (Fig. 3 and Table 1). The increase in serum glucose is a well-known response to hemorrhage, mediated by catecholamine release (Pearce and Drucker, 1987). Lactate in all three probes increased significantly with bleeding, with the greatest rise seen in blood levels. This is in general agreement with previous microdialysis measurements of lactate levels in skeletal muscle and liver during hemorrhage. (Okuda *et al.*, 1992a, 1992b) While there were trends towards increases in pyruvate, lactate/pyruvate ratio, and glycerol in blood and muscle during hemorrhage, the changes were not significant from controls.

Na⁺-K⁺ ATPase tissue levels

Table 2 summarizes the results from measurement of Na⁺-K⁺ ATPase (NKA) activities from various tissues harvested at the early decompensatory stage of shock (25% PSBVR). Activities are increased significantly ($p < 0.05$) in all tissues except liver.

The mechanism for the increase in activity is speculative at this point and may include hyperkalemia, increased levels of catecholamines, and intravascular volume depletion leading to decreased effects of ouabain-like NKA inhibitors.

Future Work/Recommended Changes

The next phase of experiments will be the measurement of cardiac contractility in addition to microdialysis during hemorrhage. These measurements will provide an assessment of the relative contributions of cardiac vs. vascular collapse as a mechanism for decompensation.

We then plan to perform experiments with tissue harvests at late decompensation (50% PSBVR) and at pre-decompensation (50% predicted peak shed blood volume) along with matched control

groups. From there we will move on to the study of animals resuscitated with crystalloid at corresponding points in early hemorrhage, early decompensation, and late decompensation.

The original proposal included the measurement of vascular smooth muscle potentials *in vitro*. The standard methodology for this technique, as practiced in numerous laboratories including ours, requires equilibration in an electrolyte bath for 90 minutes and does not allow for a true assessment of the *in vivo* potential, in the milieu of the animal at time of harvest. As such, we have discontinued these experiments from the protocol. This deletion will not change the original Statement of Work.

5. Expand current experimental protocol to include measurement of cardiac contractility. TO BEGIN MARCH 2003.

As discussed above, we will begin pilot experiments introducing cardiac contractility measurements into our standard protocol in the latter half of March 2003.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that hyperkalemia in skeletal muscle appears to precede that seen intravascularly
- Demonstrated significant deviation from equilibrium with microdialysis interstitial measurements, even for small electrolytes
- Measured tissue levels of glucose, lactate, pyruvate, and glycerol during hemorrhagic shock
- Demonstrated that ATPase activity is increased in harvested tissues of hemorrhaged animals

REPORTABLE OUTCOMES

Published Abstracts

Oliver, J. D., T. B. Bentley, J. L. Schooley, L. Chen, E. R. Morris, J. L. Atkins, and M B Pamnani. Microdialysis (μ D) measurement of interstitial potassium concentrations during hemorrhagic shock. *FASEB J.* 16:A1122, 2002.

Oliver, J. D., J. L. Atkins, J. L. Schooley, Y. Wang, T. B. Bentley, L. Ma, and M. B. Pamnani. Microdialysis (μ D) measurement of interstitial markers of hemorrhagic shock. Accepted to *FASEB J.*, 2003.

Oliver, III, J. D., J. L. Atkins, J. L. Schooley, E. R. Morris, L. Ma, T. B. Bentley, and M. B. Pamnani. Interstitial concentrations during hemorrhagic shock. Accepted to *Shock*, 2003.

Presentations

| Date | Title | Meeting |
|-----------|--|--------------------------------------|
| 24 Apr 02 | Microdialysis (μ D) Measurement Of Interstitial Potassium Concentrations During Hemorrhagic Shock | Experimental Biology New Orleans, LA |
| 11 Sep 02 | Measurement Of Interstitial And Intrahepatic | Advanced Technology |

| | | |
|-----------|--|--|
| | Electrolytes During Hemorrhagic Shock Using Microdialysis And Inductively- Coupled Plasma Mass Spectrometry (ICP- MS) | Applications for Combat Casualty Care St. Pete Beach, FL |
| 15 Apr 03 | Microdialysis (μ D) Measurement of Interstitial Markers of Hemorrhagic Shock | Experimental Biology San Diego, CA |
| 09 Jun 03 | Interstitial Concentrations during Hemorrhagic Shock | Shock Society Phoenix, AZ |

CONCLUSIONS

In our studies of the early decompensatory phase of hemorrhagic shock, we have noted significant changes in interstitial potassium levels, in serum and interstitial lactate levels, and in serum glucose concentrations. In addition, we have seen elevated NKA activity in several tissues. The elevation in potassium and NKA activity may suggest a mechanism for the etiology of hemodynamic decompensation due to severe hemorrhage, and our upcoming experiments measuring cardiac contractility should provide considerable insight into the relative contributions of cardiac versus vasodilatory collapse.

Elucidation of these mechanisms and identification of the key metabolic mediators of hemorrhagic shock should eventually result in better monitoring strategies and establishment of superior diagnostic, prognostic, and therapeutic approaches to the care of casualties.

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APPENDICES

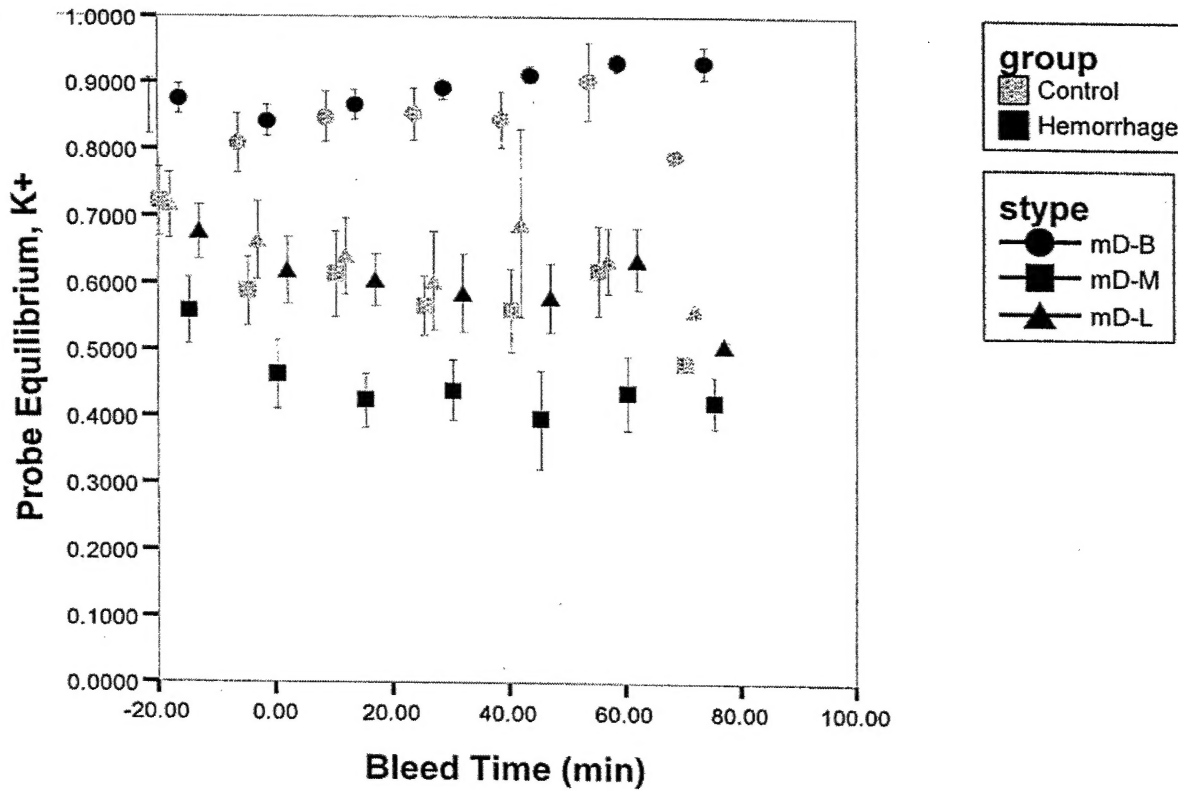


Fig. 1a. Degree of probe equilibrium for potassium as determined by Rb^+ loss from perfusate. Error bars represent \pm one S.E.M.

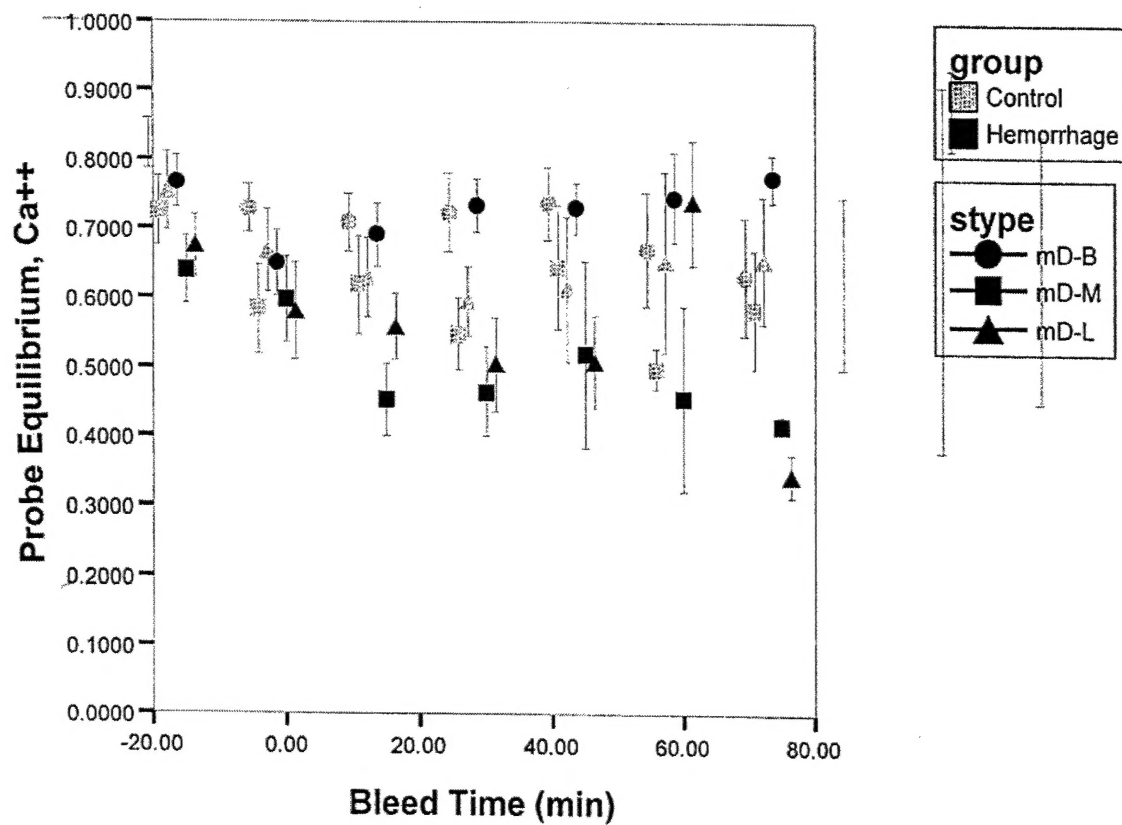


Fig. 1b. Degree of probe equilibrium for calcium as determined by ^{44}Ca loss from perfusate. Error bars represent \pm one S.E.M.

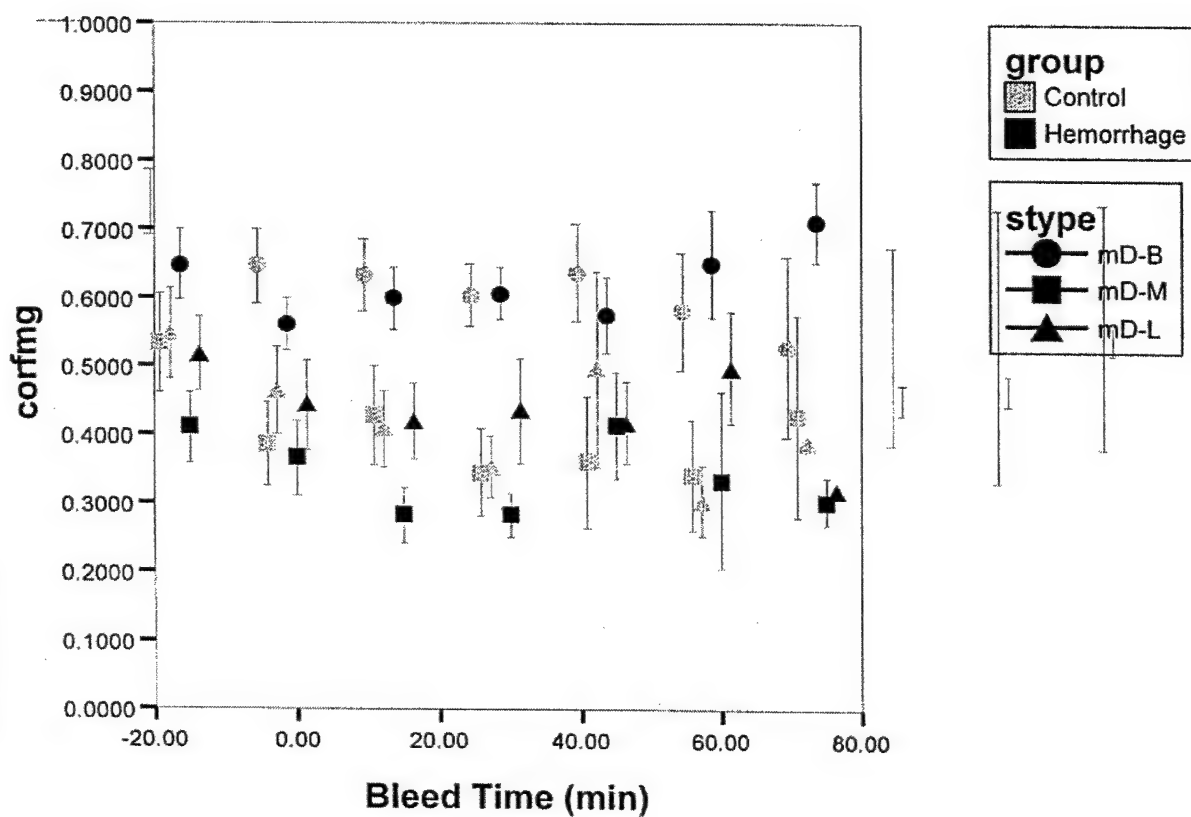


Fig. 1c. Degree of probe equilibrium for magnesium as determined by ^{26}Mg loss from perfusate. Error bars represent \pm one S.E.M.

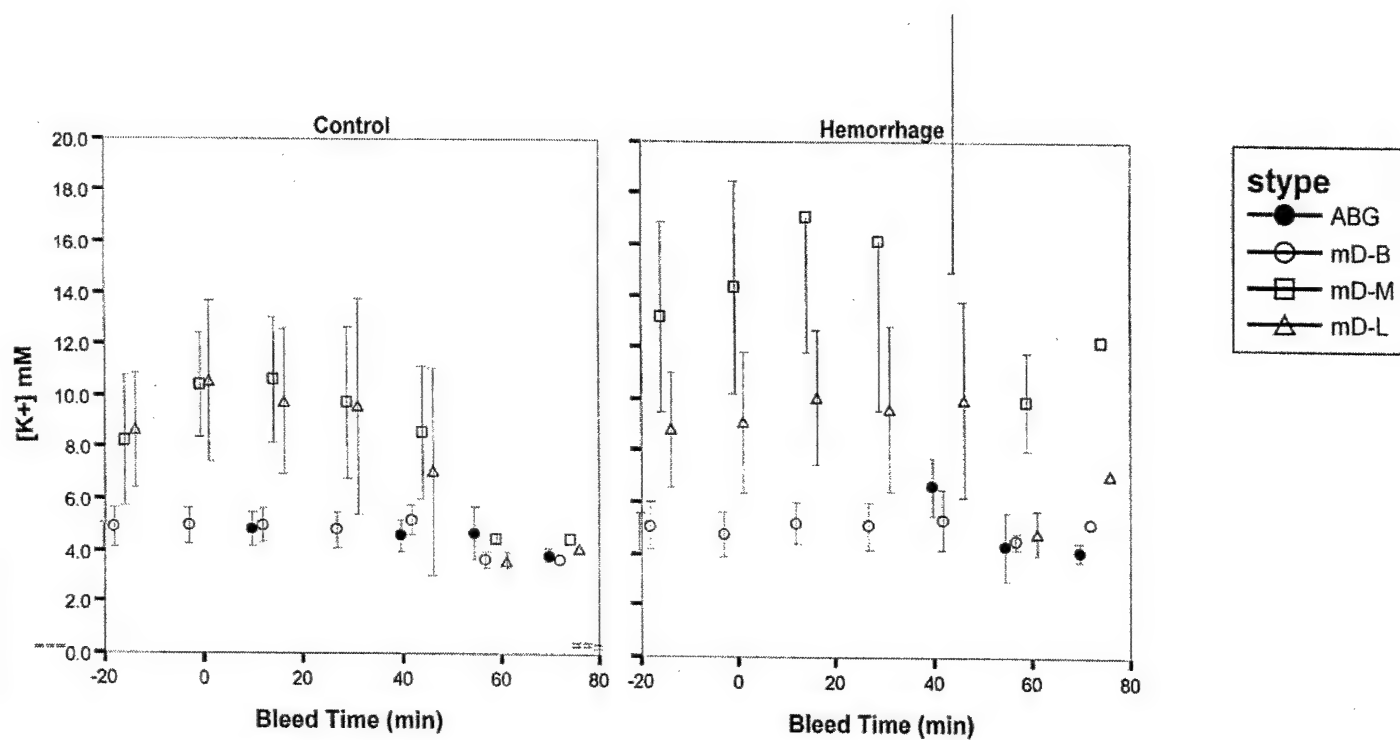


Fig. 2a. Potassium concentrations from blood probe (B), muscle probe (M), liver probe (L) and from direct arterial sampling (ABG) versus time for control and hemorrhaged animals. Error bars represent one S.E.M.

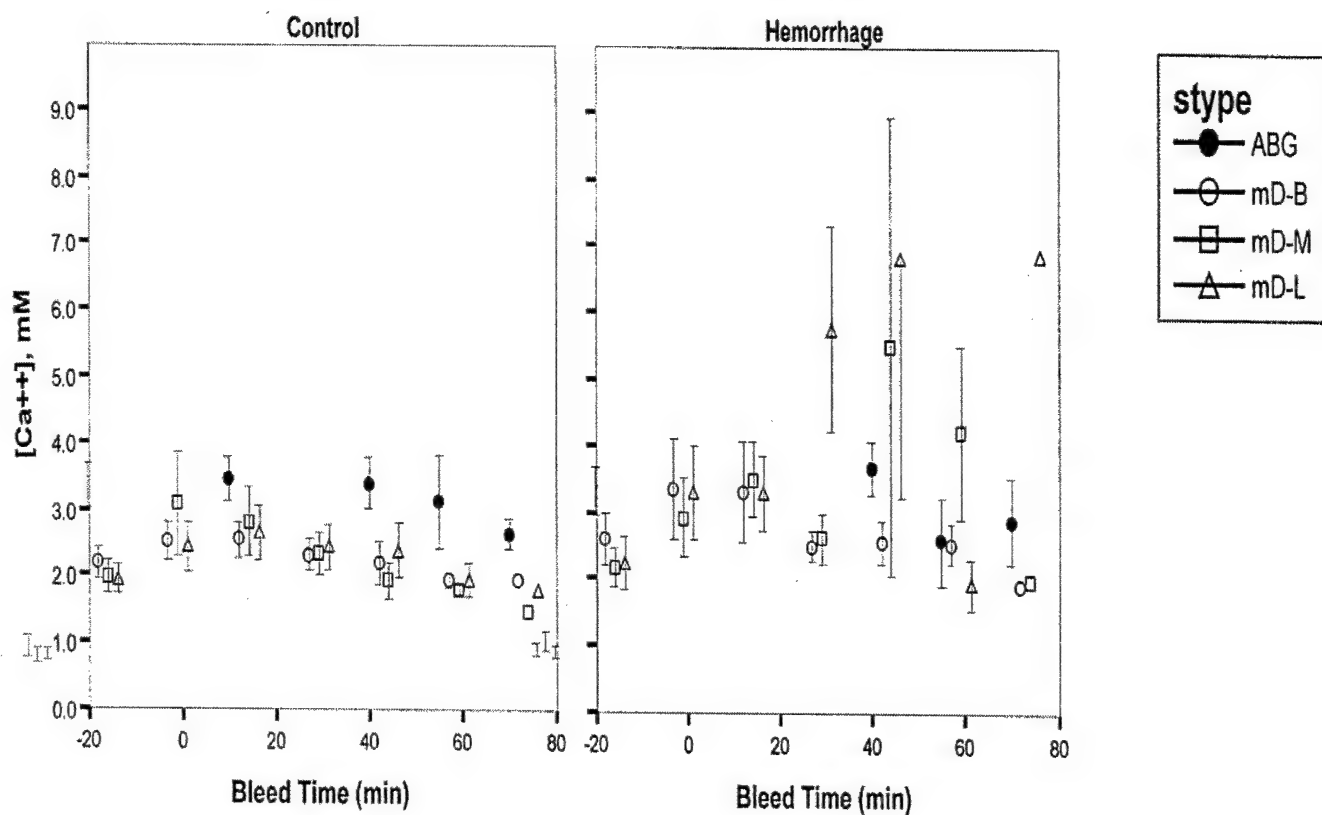


Fig. 2b. Calcium concentrations from blood probe (B), muscle probe (M), liver probe (L) and from direct arterial sampling (ABG) versus time for control and hemorrhaged animals. Error bars represent one S.E.M.

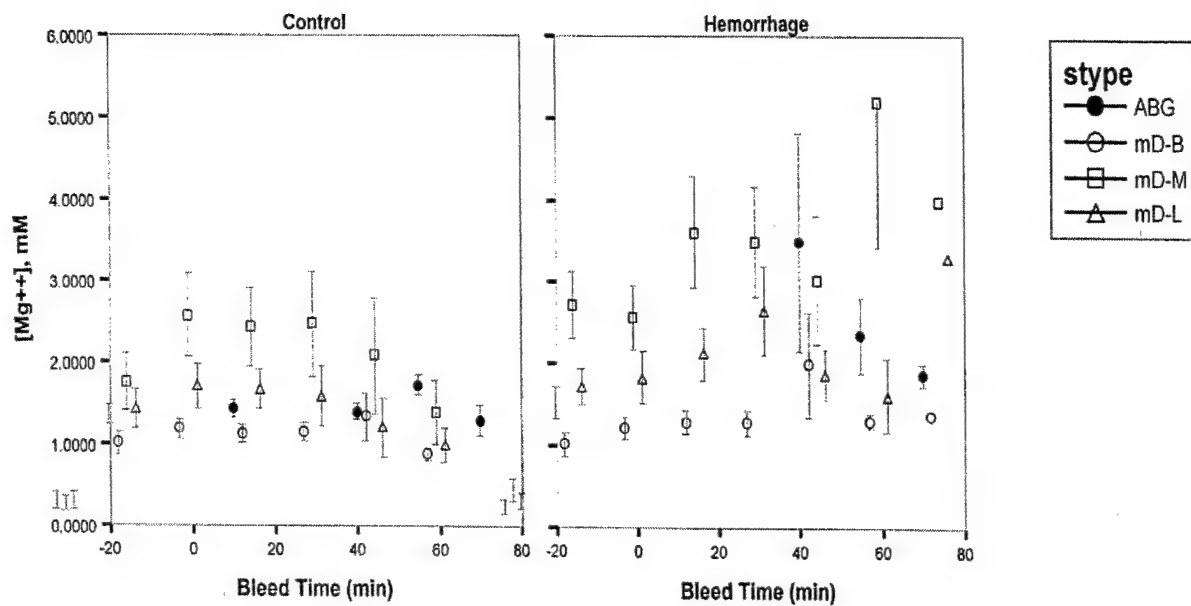


Fig. 2c. Magnesium concentrations from blood probe (B), muscle probe (M), liver probe (L) versus time for control and hemorrhaged animals. Error bars represent one S.E.M.

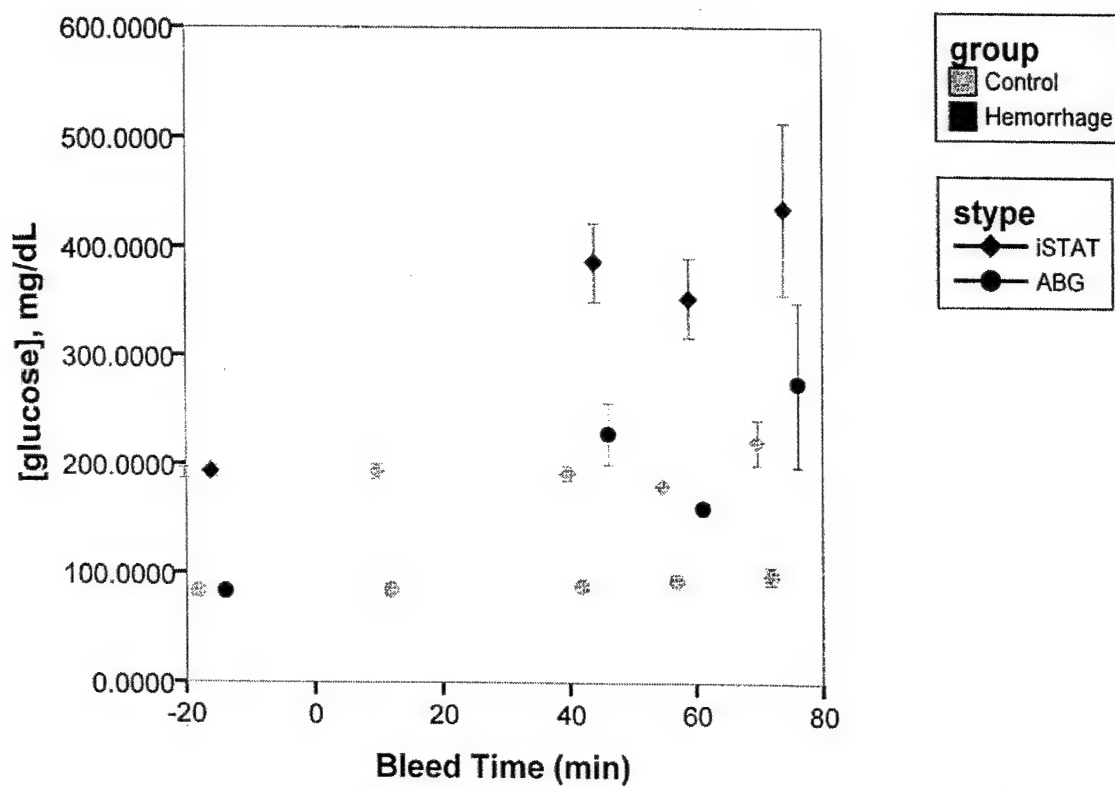


Fig 3a. Serum glucose concentrations as measured by iSTAT and glucometer (ABG) in control and hemorrhaged animals.

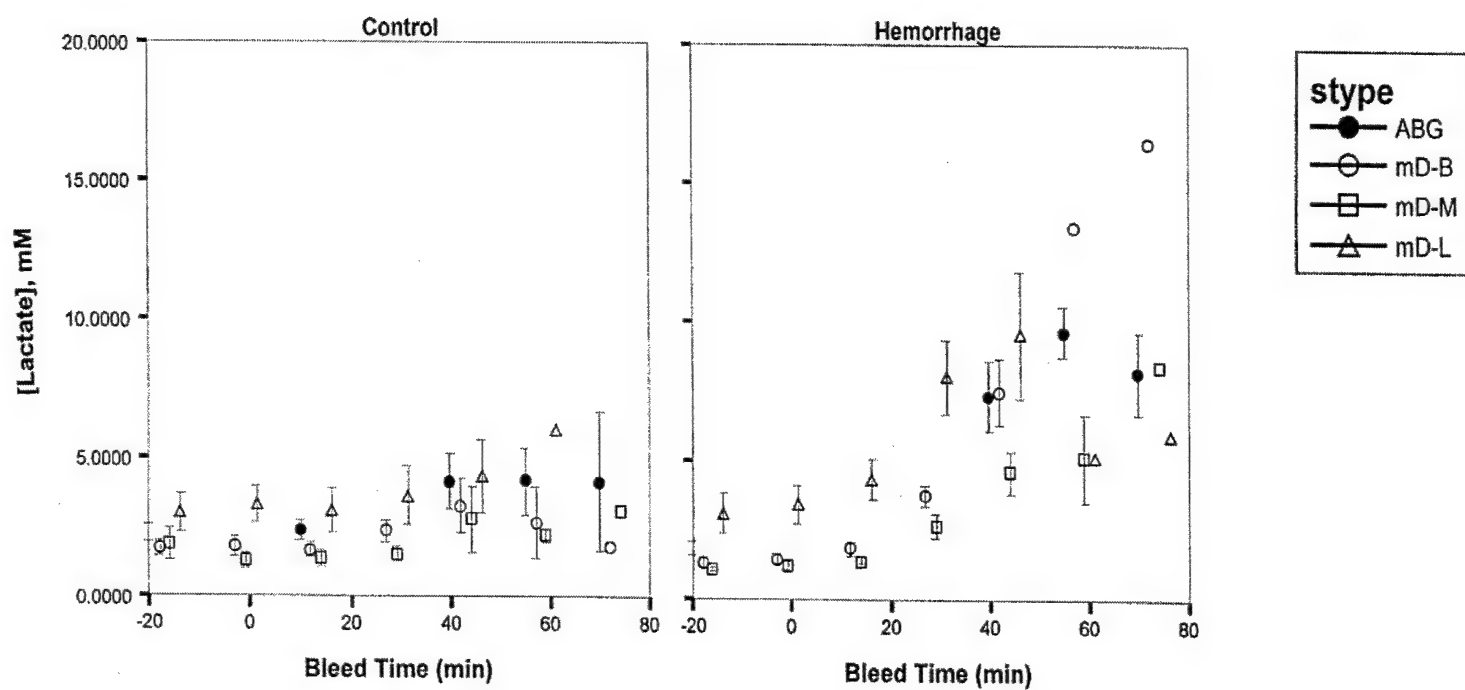


Fig 3b. Lactate concentrations in blood (B), muscle (M) and liver (L) as measured by microdialysis probes. ABG values represent values measured from direct arterial sampling.

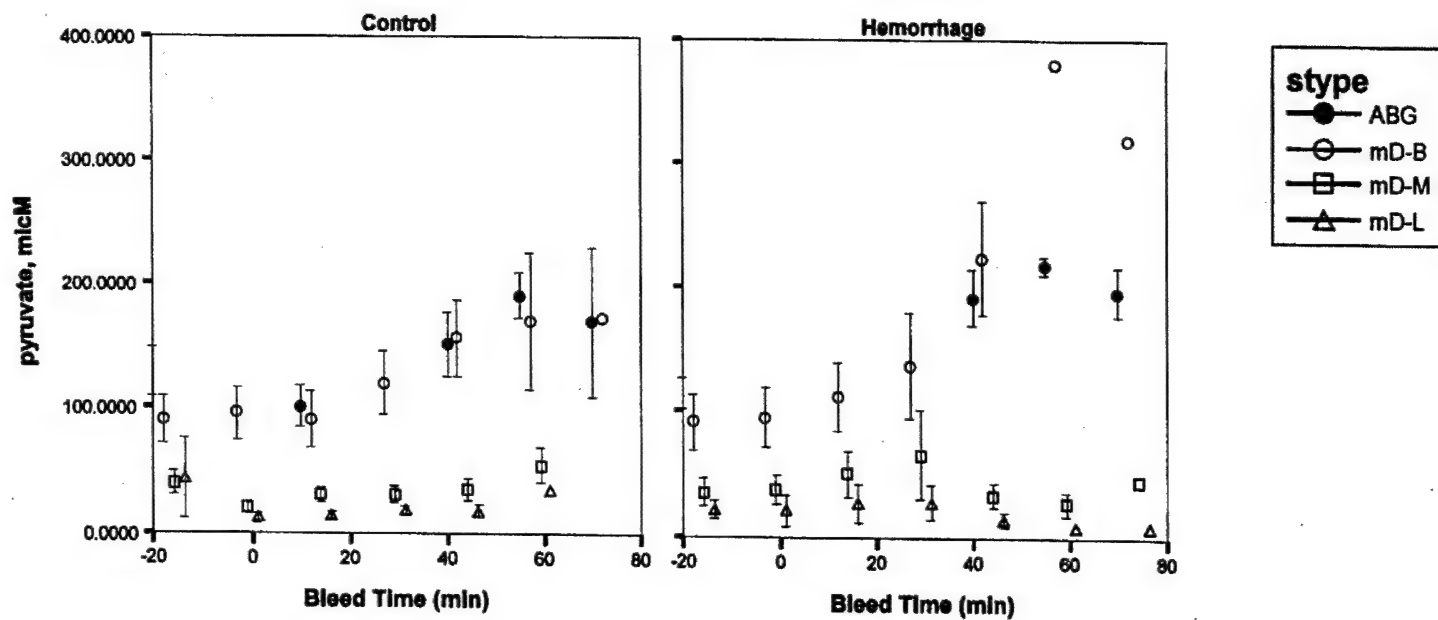


Fig 3c. Pyruvate concentrations in blood (B), muscle (M) and liver (L) as measured by microdialysis probes. ABG values represent values measured from direct arterial sampling.

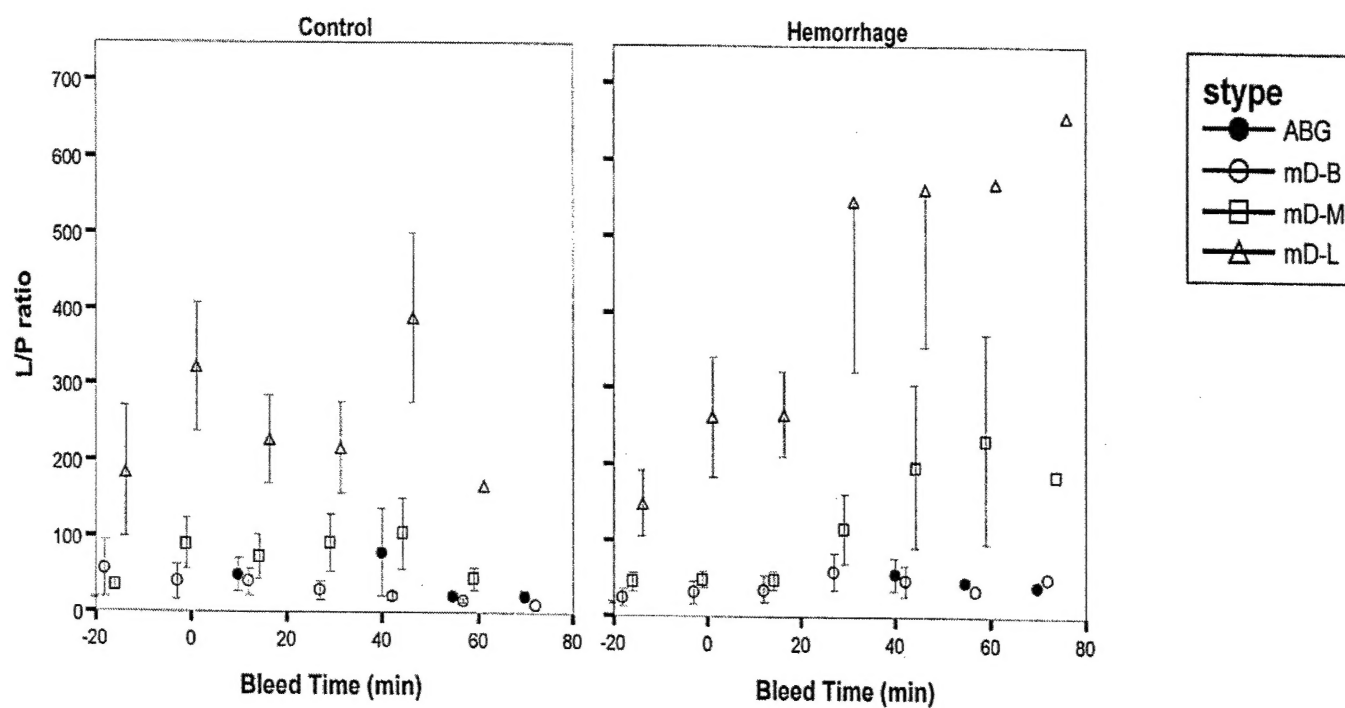


Fig 3c. Lactate-to-pyruvate ratios in blood (B), muscle (M) and liver (L) as measured by microdialysis probes. ABG values represent values measured from direct arterial samplings

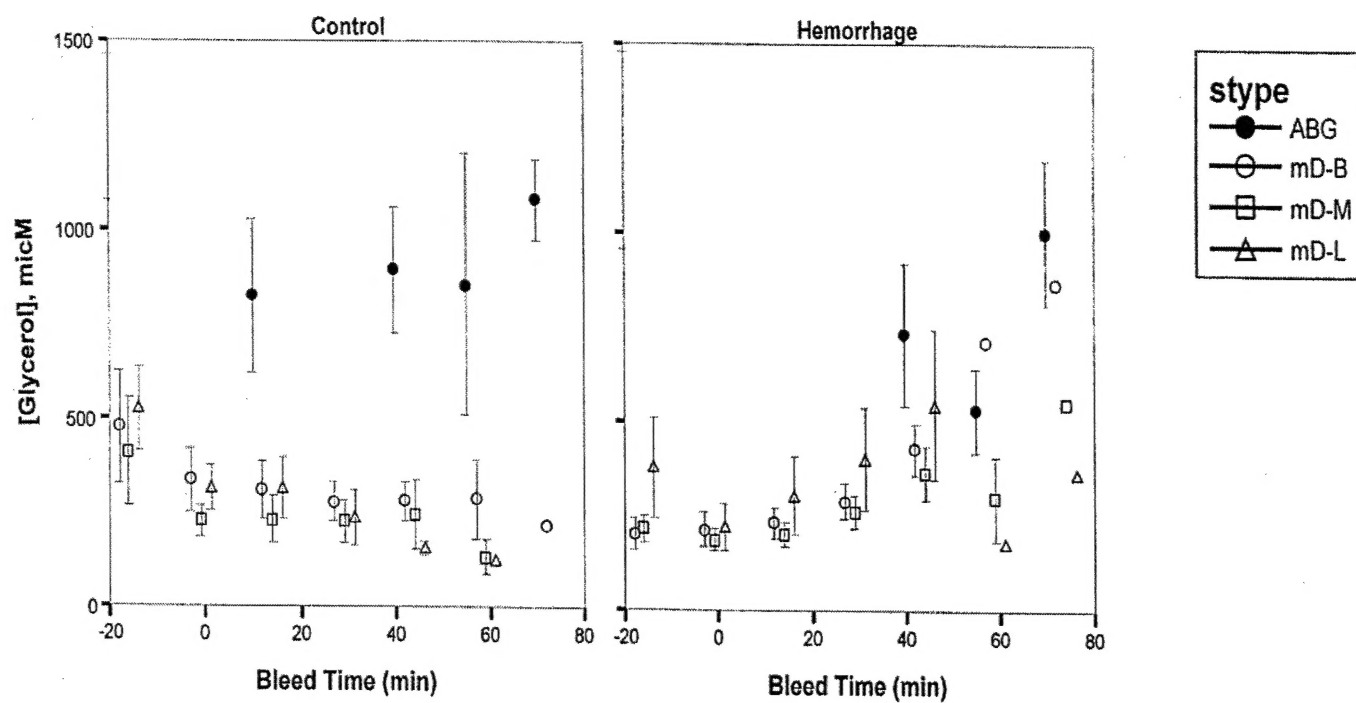


Fig 3d. Glycerol concentrations in blood (B), muscle (M) and liver (L) as measured by microdialysis probes. ABG values represent values measured from direct arterial sampling

| | Value @t=43 min hemorrhage/Baseline | | |
|------------------|-------------------------------------|------------|------------|
| | Vein | Muscle | Liver |
| K ⁺ | 1.17±0.16 | 1.98±0.45* | 0.93±0.13 |
| Ca ²⁺ | 1.28±0.14 | 1.48±0.02 | 1.26±0.05 |
| Mg ²⁺ | 1.42±0.17 | 1.32±0.24 | 0.94±0.07 |
| glucose | 2.18±0.54* | 1.46±0.15 | 1.48±0.21 |
| lactate | 6.02±0.54* | 3.64±0.39* | 2.50±0.18* |
| pyruvate | 2.91±0.42 | 1.15±0.25 | 0.82±0.18 |
| lac/pyr | 2.19±0.44 | 3.48±0.72 | 3.25±0.39 |
| glycerol | 2.17±0.41 | 1.29±0.25 | 1.41±0.35 |

Table 1. Dimensionless ratio of values at t=43 minutes of hemorrhage to baseline values. $n = 4$ for control and hemorrhage groups. Values shown \pm one S.E.M. * $p < 0.05$ compared to controls.

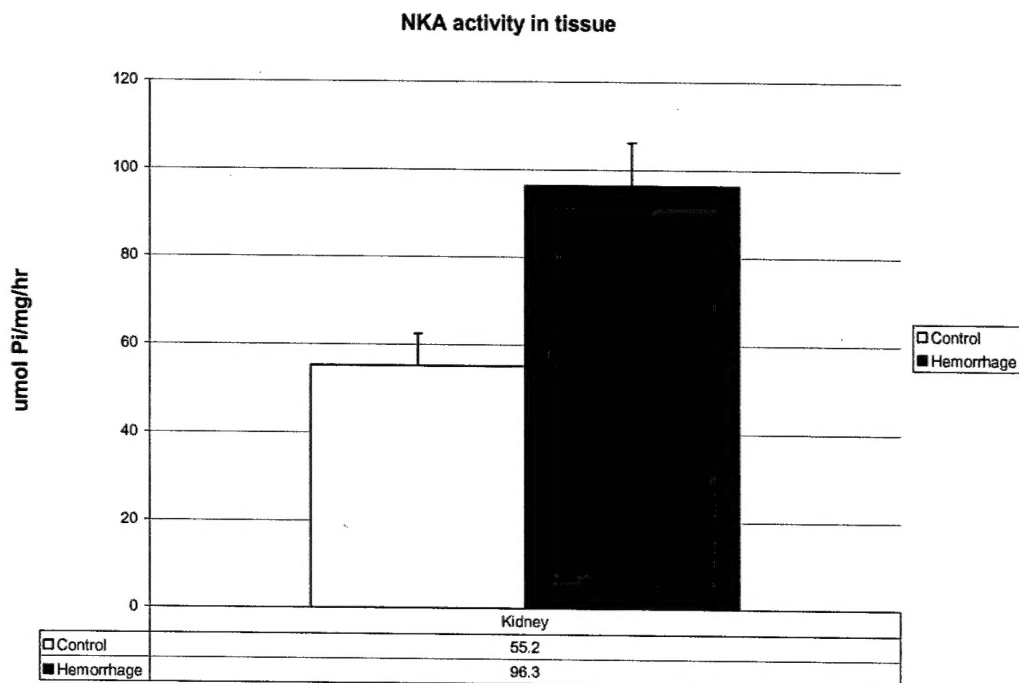
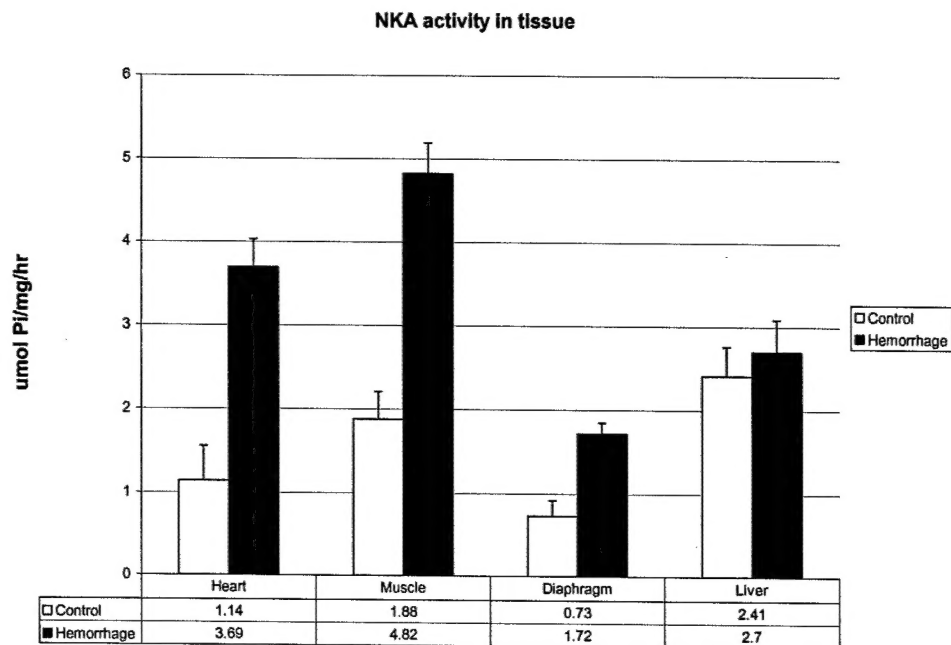


Fig 4. NKA activity in harvested tissues. Tissues were harvested at 25% peak shed blood volume return. $n = 11$ for control and hemorrhage groups. Values shown \pm S.E.M. $p < 0.05$ for all tissues except liver.